Conformational and functional significance of residue proline 17 in chicken muscle adenylate kinase

Xiangrong Sheng*, Xianming Pan, Chu Wang, Yu Zhang, Guozhong Jing

National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China

Received 19 October 2001; accepted 23 October 2001

First published online 5 November 2001

Edited by Pierre Jolles

Abstract The effect of mutation proline 17 on the multiple conformations and catalytic function in chicken muscle adenylate kinase (AK) has been studied. The substitution of proline 17 with glycine or valine altered the distribution of multiple conformations. Compared with the wild-type enzyme, the P17G and P17V mutants contained decreased fraction of minor conformer from 18% to 9% and 11%, respectively. Due to the mutation, the enzyme showed lower secondary structural content, poorer affinity to substrates or substrate analogues, and reduced catalytic efficiency. The results revealed the significance of proline 17 in the conformation and function of AK. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Adenylate kinase; Proline residue; Multiple conformation; Kinetics

1. Introduction

Proline is unique among the amino acids with its five-member ring. Thus, proline residues play specific structural roles: as N-terminal caps to α-helices, as helix termination signals, as corner residues in β-turn sequences. Moreover, *cis* and *trans* conformations of Xaa–Pro bonds have comparable energies, leading to a 10–30% population of *cis* Xaa–Pro in the unfolded assemble and an about 6% frequency of *cis* Xaa–Pro bonds that are in native proteins [1,2]. As a result, some proteins show structural heterogeneity in the native state due to isomerization about a Xaa–Pro peptide bond [3,4]. According to the NMR studies of staphylococcal nuclease (SNase), the structural heterogeneity can be ascribed to the isomerizations about the Lys116–Pro117 and His46–Pro47 peptide bonds [5]. The heterogeneity in the native state was eliminated by the substitution of glycine for Pro117. The isomerizations about these two peptide bonds of SNase occur independently and produce the four different native conformers [6].

The role of Xaa–Pro peptide bond isomerizations in the kinetics of protein folding has been investigated thoroughly, and it is now widely accepted that such isomerizations are often the reason for slow steps in protein folding reactions [7]. In addition, it has been suggested that this isomerization can play an important role in protein function; for example, a proline may be actively involved in the regulation of transmembrane proteins such as the sodium pump, by having *cis*/*trans* isomerization synchronous with ion translocation [8].

The relationship between multiple conformations and protein function is of great interest nowadays. It has been believed that the studies on the affection of multiple conformations of proteins will shed light on the understanding of protein folding mechanism both in thermodynamic and kinetic processes.

Adenylate kinase (AK, EC 2.7.4.3) is a ubiquitous multiple-domain enzyme that controls the cellular energy balance by catalyzing the transfer of a phosphoryl group from ATP to AMP: MgATP+AMP⇔MgADP+ADP [9]. Close to 20 crystal structures of various AKs have been solved thus far [10]. The enzyme contains two distinct nucleotide binding sites: the MgATP site, which binds MgATP and MgADP, and the AMP site, which is specific for AMP and uncomplexed ADP [11]. The catalytic mechanism and the substrate-induced conformational changes of AK have been the subject of a number of investigations [12–16]. We previously observed that at least two conformational forms of rabbit muscle AK with different binding ability to fluorescence probe, 8-anilino-1-naphthalene sulfonic acid (ANS) exist in equilibrium in solution. The ratio of the two conformers was 70:30%, and the major form bound rapidly to ANS whilst the minor form had to convert to the major form before it bound to ANS [13]. Further study on the functional meaning of multiple conformations may improve our understanding of the catalytic property and folding process for AK.

It was suggested that the conformational changes in AK be involved in proline isomerization [14,17,18]. In this work, proline 17 of chicken muscle AK has been replaced by glycine or valine using site-specific mutagenesis to investigate the structure and functional role of the proline residue. The chicken muscle cytoplasmic enzyme is a single polypeptide of 194 amino acid residues ($M_r \approx 22\,000$ Da), and sequence comparisons indicate nearly 93% homology with several other muscle-derived cytoplasmic AK species [19]. Proline 17 is located in a highly conserved glycine-rich, flexible polar loop (Gly^{15}-Gly-Pro-Gly-Ser-Gly-Lys-Gly^{22}), which is assigned to the part of the MgATP binding and ANS binding domain [11,20]. The results demonstrated that the mutation at proline 17 altered the distribution of multiple conformers, the substrate and inhibitor binding ability and the catalysis efficiency of AK.

*Corresponding author. Fax: (86)-10-64872026.
E-mail address: xrsheng@sun5.ibp.ac.cn (X. Sheng).

Abbreviations: AK, adenylate kinase; ANS, 8-anilino-1-naphthalene sulfonic acid; Ap_5A, P_1,P_5-bis(adenosine-5′-)pentaphosphate

0014-5793/01/$20.00 © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(01)03092-7

2. Materials and methods

2.1. Chemicals

Adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide, reduced (NADH), phosphoenolpyruvate (PEP), P_1,P_5-bis(adenosine-5′-)pentaphosphate (Ap_5A), ANS, hexokinase and pyruvate kinase were from Sigma (St. Louis,MO, USA), lactate dehydrogenase from Roche and glucose-6-phosphate-dehydrogenase from Boehringer Mannheim (Indianapolis, IN, USA).

2.2. Plasmid construction, protein expression and purification

Plasmid pBV-cak1 for expression of wild-type chicken muscle AK was constructed as described by Jing et al. [21]. The synthetic primers 5′-TGGGTGGCGGCGGCTCA-3′ and 5′-TGGGTGGCGTCGGCTCA-3′ were used to amplify the mutant P17G and P17V AKs, respectively, in which the mutation positions were underlined in the oligonucleotide sequence, the CCC (proline) codon was altered to a GGC (glycine) or a GTC (valine) codon. The expression plasmid pBV221-cak1 was used as a template and the site-directed mutagenesis was performed by PCR. DNA fragments carrying the point mutation were subcloned into the plasmids pBV221 (for P17G mutant) or pET-3a (for P17V mutant) using the protocol as described previously. The full-length wild-type AK, P17G and P17V mutant gene sequencing was performed with an autosequencer (ABI PRISM 377XL DNA Sequencer, Perkin-Elmer, Norwalk, CT, USA) to ensure that no undesirable mutations had occurred. Automated Edman degradation technique was used to determine the amino-terminal protein sequence of P17G mutant. Overnight culture of *Escherichia coli* (DH5α) cells containing the respective plasmid for expression of wild-type and P17G mutant AK was added at 37°C to 2 l of Luria broth (LB) medium plus ampicillin (50 μg/ml). At $OD_{600} = 0.5$, expression was induced with a temperature upshift to 42°C, and the cells were grown at 42°C for an additional 5 h. P17V mutant was expressed in the BL21(DE3) strain of *E. coli*. Protein expression was induced in LB culture with 0.4 mM isopropyl-β-thiogalactose (IPTG) when the OD_{600} of the culture reached 0.8. After an additional 3–4 h of incubation at 37°C, the cells were collected by centrifugation. The data indicated that part of wild-type and total P17G and P17V mutant AK were present in the insoluble fraction, i.e. contained in cell inclusion bodies. The purified inclusion bodies were obtained as reported by Gross et al. [22].

The inclusion bodies were dissolved in 30 ml 6 M urea in 25 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol, by stirring overnight at 4°C. The supernatant of denatured proteins was subjected to cation exchange chromatography on POROS HS/M column (Boehringer, Mannheim, Germany), which was equilibrated with the above-mentioned buffer and 6 M urea and eluted with a linear gradient of NaCl from 0 to 0.5 M at a flow rate of 2 ml/min. The pooled fractions were dialyzed against 50 mM Tris–HCl, pH 7.5, 0.15 M NaCl and 5 mM β-mercaptoethanol. The supernatant was pelleted with 90% saturated $(NH_4)_2SO_4$ and applied to Sepharcryl S-100 molecular size exclusion chromatography. The collected fractions were detected by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the presence of a single band of AK protein was confirmed.

2.3. The absorbance, CD and fluorescence spectra

The absorbance spectra during the ultra-violet range were recorded on a Shimazu UV-2501 spectrometer. CD experiments were performed on a Jasco J-720 spectropolarimeter using a thermostatic cuvette with 1 mm path length at 25°C. The baselines due to the buffer (50 mM Tris–HCl, pH 7.5, 5 mM β-mercaptoethanol) were subtracted from the spectra.

The intrinsic fluorescence spectra and the time course of ANS binding to AK fluorescence were measured on a Shimazu RF-5301PC spectrofluorophotometer at 25°C using a 1 cm thermostatic cuvette. In the intrinsic fluorescence emission spectra measurements, the excitation wavelength was set at 295 nm and the slit widths were 5 nm. To record the time course of ANS binding to AK, the excitation and emission wavelengths were separately set at 378 nm and 478 nm, respectively, and the slit widths were set to 5 nm.

Protein concentration was 5 μM and ANS concentration was 200 μM. The buffer contained 50 mM Tris–HCl, pH 7.5, and 2 mM β-mercaptoethanol. Buffer plus ANS controls containing no protein were subtracted automatically.

2.4. Enzyme assay and kinetics

The rate of the forward reaction (MgATP+AMP → MgADP+ADP) was measured by following the oxidation of NADH at 340 nm coupling with pyruvate kinase and lactate dehydrogenase. Measurements of the velocity of the backward reaction (MgADP+ADP → MgATP +AMP) were performed by monitoring the reaction of NADP at 340 nm in a coupled enzyme solution with hexokinase and glucose-6-phosphate dehydrogenase [14].

To obtain kinetic parameters, enzyme activity was measured as the concentration of either nucleotide was varied. In the case of wild-type AK, the apparent K_m for MgATP (AMP) was determined as the concentration of AMP (MgATP) was 2 mM and Mg free was 1.5 mM. The concentrations of MgADP and ADP were 0.2 mM and 0.5 mM, respectively, which were used to determine the apparent K_m for ADP and MgADP. As for the P17G mutant AK, the concentrations of the fixed nucleotides, i.e. MgATP or AMP, were 5 mM and Mg free was 1 mM, the varied concentrations were changed in the region of 0.1–2.0 mM. In the backward reaction, the fixed concentrations for MgADP and ADP were 5 mM and 2 mM, respectively.

The inhibition of Ap_5A for wild-type and P17G mutant chicken muscle AK was assayed in the forward and backward reaction by adding various amounts of $MgAc_2$, ATP, AMP, ADP and Ap_5A to the standard reaction mixture.

All reactions were measured in a Shimazu UV2501 spectrometer at 25°C. An adequate amount of enzyme that had been diluted with 50 mM Tris–HCl containing 2 mM β-mercaptoethanol and 7.6 μM bovine serum albumin was added to the assay mixture. The final concentrations of wild-type and mutant P17G were 0.5 μM and 4.3 μM in all kinetic measurements, respectively. One unit of enzyme activity is defined as the amount which produces 1 μmol ADP in 1 min.

3. Results

3.1. Site-directed mutagenesis and purification of expressed protein

The entire gene sequencing of wild-type, P17G and P17V of AK showed the correct DNA sequences were obtained. N-terminal sequencing of the mutants P17G and P17V was performed with PVDF membrane-blotted material by automated Edman degradation. The N-terminal sequence was assigned to be Ser-Thr-Glu-Lys-His-His for P17G, which was identical to that predicted from the cDNA sequence except that the NH_2-terminal methionine was absent and agreed to the previous report [19]. The mutant proteins migrated as expected for a protein with a molecular weight of 22 000 Da. The wild-type enzyme and both mutants were prepared from refolding of inclusion bodies. The P17G and P17V mutants possessed the same chromatographic elution patterns as the wild-type enzyme.

Table 1
Apparent kinetic parameters for wild-type and P17G mutant AK

Protein	K_m AMP (mM)	K_m MgATP (mM)	K_m ADP (mM)	K_m MgADP (mM)	V_{max} (units/mg)[a]
Wild-type	0.17	0.15	0.0035	0.21	1623
P17G mutant	3.4	5.1	0.0098	1.1	650

[a]The V_{max} value was obtained when the concentration of MgATP was varied.

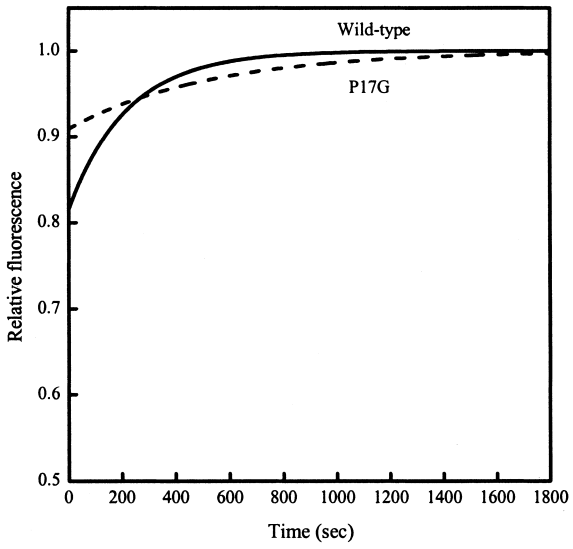

Fig. 1. Time course of fluorescence change with ANS binding to wild-type and P17G mutant chicken muscle AK. Straight line: wild-type AK; dashed line: P17G mutant AK. The excitation and emission wavelengths were separately set at 378 nm and 478 nm, respectively, and the slit widths were set to 5 nm. Protein concentration was 5 μM and ANS concentration was 200 μM. The buffer contains 50 mM Tris–HCl, pH 7.5, and 2 mM β-mercaptoethanol.

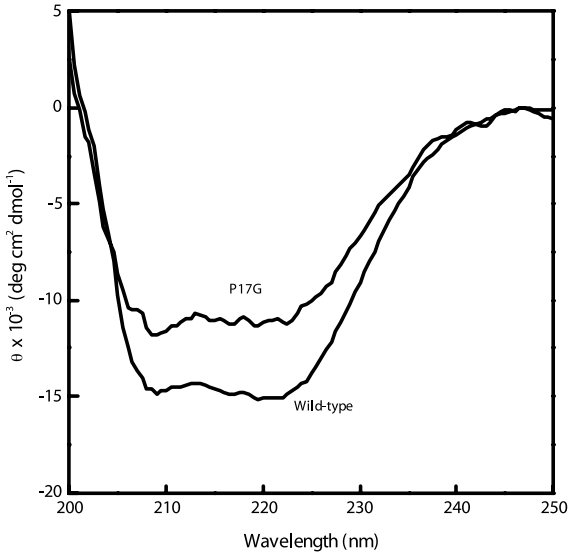

Fig. 2. CD spectra of wild-type, P17G mutant chicken muscle AK. All proteins were measured at a protein concentration of 5 μM in 50 mM Tris–HCl, pH 7.5, 5 mM β-mercaptoethanol, at 25°C using an optical path of 0.1 cm.

3.2. Changes of conformational distribution of P17G probed by ANS fluorescence

In the previous study of this laboratory, ANS binding fluorescence was found to be a sensitive and effective measurement to detect the multiple conformations of rabbit muscle AK in solution. The ratio of the relative amplitudes of different kinetic phases represented the ratio of individual conformers and the rate constant of the slow phase of ANS binding indicated the converting rate between different conformers at some extent [13]. The replacement of proline 17 by glycine of the chicken muscle AK alters the time course of ANS binding fluorescence as compared to the wild-type enzyme. The fluorescence changes along with ANS binding displayed two distinguished kinetic phases: the fast phase completed in the dead time of manual mixing, and the slow phase reached the constant value in about 30 min, as shown in Fig. 1. The fluorescence traces for the slow phase could be fitted to a monoexponential function. The fitted rate for P17G mutant (0.00185 s^{-1}) is obvious slower than that for the wild-type enzyme (0.00451 s^{-1}), as well as the relative amplitude of the slow phase ANS fluorescence of P17G mutant (9%) was declined to about 1/2 that of the wild-type (18%).

3.3. Conformational changes of AK due to proline 17 to glycine mutation

The absorbance and intrinsic fluorescence spectra of the mutant and wild-type AK showed that the P17G mutant did not deviate significantly from those of the wild-type. However, the CD spectra of wild-type and mutant enzyme indicated the considerable loss of secondary structure content due to the replacement of proline 17 by glycine. As can be seen from Fig. 2, the secondary structure content of P17G mutant was decreased to about 75% of that of wild-type AK.

3.4. Comparison of kinetic parameters for wild-type and mutant P17G AK

A complete summary of kinetic parameters that were calculated using the previous reported method [14] was given in Table 1. The mutation of proline 17 to glycine of the chicken muscle AK resulted in a 2.5-fold reduction in the observed maximal velocity (in the forward reaction) as compared to the wild-type enzyme. The apparent K_m values of the P17G mutant for AMP and MgATP were increased approximately by 20- and 34-fold, respectively. The results suggest that the mutation at residue proline 17 influenced both the binding of substrates and the catalytic efficiency of the enzyme, which had a slight difference with a previous report by Tagaya et al. [23]. Furthermore, proline 17 played a more important role for the binding of substrates MgATP and AMP in the forward reaction.

3.5. Ap_5A inhibition effect on the wild-type and mutant P17G AK

Ap_5A is a bisubstrate analogue of AK. It was reported by Kuby et al. that Ap_5A acted as a competitive inhibitor for the forward reaction and a non-competitive inhibitor for the backward reaction [24]. However, the re-examined data showed that Ap_5A acted as a mixed non-competitive inhibitor for the backward reaction, which agreed with origin figures shown in the paper of Kuby et al. [14,24]. In the present

Table 2
Inhibition constants of Ap_5A with wild-type and P17G mutant AK

Protein	K_i AMP (M)	K_i MgATP (M)	K_i ADP (M)	K_i MgADP (M)
Wild-type	2.05×10^{-7}	2.32×10^{-7}	1.26×10^{-8}	1.15×10^{-7}
P17G mutant	1.10×10^{-5}	8.53×10^{-6}	3.66×10^{-6}	2.70×10^{-6}

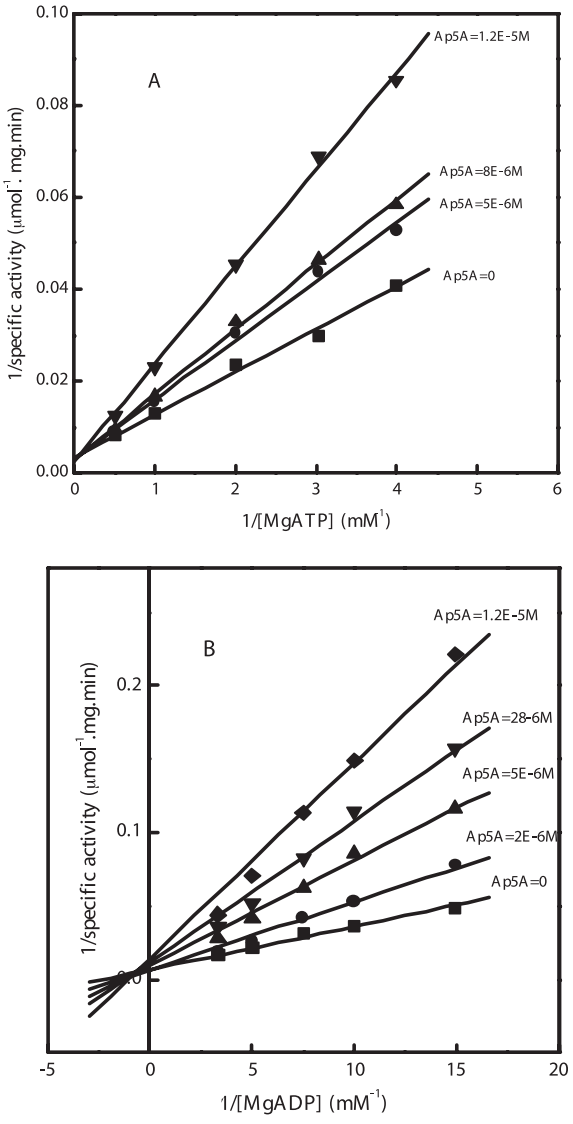

Fig. 3. Inhibition pattern of Ap_5A for P17G chicken muscle AK. A: In the forward reaction. B: In the backward reaction.

study, similarly the inhibition pattern of Ap_5A for the chicken muscle AK was different between various substrates. The results were shown in Fig. 3, which demonstrated clearly that the inhibition was competitive with respect to the forward reaction substrates: MgATP and AMP; and was mixed non-competitive with respect to the backward reaction substrates: MgADP and ADP. For both the wild-type and P17G mutant AK, the Ap_5A inhibition patterns seemed alike except with different effective inhibitor concentration regions.

The parameters K_i were obtained according to the previous study [24] and listed in Table 2. The increased concentrations of Ap_5A necessary to inhibit the P17G enzyme indicated a significantly reduced affinity for the bisubstrate analogue.

3.6. Study on the mutant of P17V AK

In the above experiment, a highly conformationally restricted proline is mutated to a glycine in a loop region, which is already rich in glycine. It may well be that the entropic effect of incorporating yet another flexible residue into the loop is sufficient to destabilize the protein and cause the effects observed, i.e. loss of structural content, reduced ligand affinity, and lower catalytic efficiency [25]. To test this issue, an alternative mutant of P17V (replace proline 17 by valine) was constructed and studied. The mutant P17V shows the similar effect on the conformational and functional changes as mutant P17G. The specific activity and secondary structure content of P17V are decreased to about 10% and 60% of that of the wild-type, respectively. The time course of ANS binding to P17V displayed also a burst phase and a slow phase with a ratio of amplitude of burst phase to slow phase 89:11. This result excludes the possibility that the observed conformational and functional changes of P17G are caused by the unique effect of glycine as a flexible residue and potent *cis* conformation contributor. The attempt to obtain the kinetics and Ap_5A inhibition parameters of P17V failed, since the specific activity of P17V is too low.

4. Discussion

The effects of proline mutations on the structure and function of the protein are remarkably different among the individual proteins or different among the mutation sites in the same protein depending on structural context. The local perturbation resulting from the removal of proline residues significantly altered the stability and folding kinetics in some proteins [6]. SNase is known to exhibit proline isomerization in not only the unfolded but also the native state, and this gives rise to structural heterogeneity in the native state. The P117G mutation increased the protein stability and affected both refolding and unfolding kinetics [6]. On the other hand, in some cases, no gross structural changes occurred upon proline substitution even in those proteins that had the multiple conformations arising from proline isomerization [4]. For calbindin D_{9K}, structural perturbations due to proline isomerization were localized and the globular conformations of the two forms were very similar [4].

In the case of AK from chicken muscle, proline 17 is located in a highly conserved glycine-rich, flexible polar loop (Gly^{15}-Gly-Pro-Gly-Ser-Gly-Lys-Gly^{22}), which together with the more distal residues Lys27, Phe183 and Leu190 appears to define components of the MgATP binding domain [20]. Furthermore, this loop remarkably moves during the transition between two crystal forms [11,12]. The results in this report showed that the substitution of proline 17 in AK remarkably affected the secondary structure content, the multiple conformational distributions, enzyme catalytic activity and the affinity to the inhibitor Ap_5A.

As has been determined by site-specific mutagenesis experiments, when proline 17 is mutated to glycine or valine, the fraction of fast and slow phase of ANS binding fluorescence is changed significantly. Meanwhile, catalytic activity decreased 2.5-fold for P17G and 10-fold for P17V, and the affinity of the enzyme for both substrates as well as the bisubstrate analogue Ap_5A is decreased substantially (see Tables 1 and 2). The present results suggest that the replacement of proline 17 by glycine or valine causes conformational alterations, and even subtle alterations in the local environment may significantly influence the distribution of multiple conformers, consequently influence the function of the enzyme. The proline 17 residue is essential for retaining the proper conformation and positioning the substrate binding residues to achieve high substrate affinity and high activity.

As a summary, proline 17 plays an essential role on keeping

the multiple conformations of AK. The full expression of catalytic activity of AK relies on the proper conformation distribution and discrete substrate binding conformation.

Acknowledgements: The present study was supported in part by the Project 30000029 from the National Natural Science Foundation of China, the 973 Project G1999075606 from the China Commission for Science and Technology and the Project STZ-01-09 from Chinese Academy of Sciences. We thank Professor C.L. Tsou of the Institute of Biophysics, Chinese Academy of Sciences, for many helpful suggestions.

References

[1] Forood, B., Feliciano, E.J. and Nambiar, K.P. (1993) Proc. Natl. Acad. Sci. USA 90, 838–842.
[2] Pieto, J. and Serrano, L. (1997) J. Mol. Biol. 274, 276–288.
[3] Fox, R.O., Evans, P.A. and Dobson, C.M. (1986) Nature 320, 192–194.
[4] Chazin, W.J., Kodel, J., Drakenberg, T., Thulin, E., Brodin, P., Grunstrom, T. and Forsen, S. (1989) Proc. Natl. Acad. Sci. USA 86, 2195–2198.
[5] Loh, S.N., McNemar, C.W. and Markley, J.L. (1991) in: Techniques in Protein Chemistry Vol. 2, pp. 275–282, Academic Press, San Diego, CA.
[6] Ikura, T., Tsurupa, G.P. and Kuwajima, K. (1997) Biochemistry 36, 6529–6538.
[7] Kim, P.S. and Baldwin, R.L. (1990) Annu. Rev. Biochem. 59, 631–660.
[8] Vanhoof, G., Goosens, F., De Meester, I., Hendriks, D. and Scharpe, S. (1995) FASEB J. 9, 736–744.
[9] Noda, L. (1973) in: The Enzymes (Boyer, P.D., Ed.), Vol. 8, pp. 279–305, Academic Press, New York.
[10] Vorherin, C., Schlauderer, G.J. and Schultz, G. (1995) Structure 3, 483–490.
[11] Pai, E.F., Sachsenheimer, W., Schirmer, R.H. and Schulz, G.E. (1977) J. Mol. Biol. 114, 37–45.
[12] Berry, M.B., Meador, B., Bilderback, T., Liang, P., Glaser, M. and Philips Jr., G.N. (1994) Proteins Struct. Funct. Genet. 19, 183–198.
[13] Zhang, H.J., Sheng, X.R., Pan, X.M. and Zhou, J.M. (1998) J. Biol. Chem. 273, 7448–7456.
[14] Sheng, X.R., Li, X. and Pan, X.M. (1999) J. Biol. Chem. 274, 22238–22242.
[15] Shapiro, Y.E., Sinev, M.A., Sineva, E.V., Tugarinov, V. and Meirovitch, E. (2000) Biochemistry 39, 6634–6644.
[16] Berry, M.B. and Phillips Jr., G.N. (1998) Proteins Struct. Funct. Genet. 32, 276–288.
[17] Sheng, X.R., Zhang, H.J., Pan, X.M. and Zhou, J.M. (1997) FEBS Lett. 413, 429–432.
[18] Li, X. and Pan, X.M. (2000) FEBS Lett. 480, 235–238.
[19] Tanizawa, Y., Kishi, F., Kaneko, T. and Nakazawa, A. (1987) J. Biochem. 101, 1289–1296.
[20] Okajima, T., Tanizawa, K., Yoneya, T. and Fukui, T. (1991) J. Biol. Chem. 266, 11442–11447.
[21] Jing, E.X., Zhou, B., Luo, J., Zhang, H.J. and Jing, G.Z. (1997) Prog. Biochem. Biophys. 24, 525–528.
[22] Gross, M., Wyss, M., Furter-Graves, E.M., Wallimann, T. and Futer, R. (1996) Protein Sci. 5, 320–330.
[23] Tagaya, M., Yagami, T., Noumi, T., Futai, M., Kishi, F., Nakzawa, A. and Fukui, T. (1989) J. Biol. Chem. 264, 990–994.
[24] Kuby, S.A., Hamada, M., Gerber, D., Tsai, W.C., Jacobs, H.K., Cress, M.C., Chua, G.K., Fleming, G., Wu, L.H., Fischer, A.H., Frischat, A. and Maland, L. (1978) Arch. Biochem. Biophys. 187, 34–52.
[25] Matthews, B.W., Nicholson, H. and Becktel, W.J. (1987) Proc. Natl. Acad. Sci. USA 84, 6663–6667.